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In Vitro Anti-*Plasmodium falciparum* Properties of the Full Set of Human Secreted Phospholipases A_2

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We have previously shown that secreted phospholipases A_2 (sPLA$_2$s) from animal venoms inhibit the *in vitro* development of *Plasmodium falciparum*, the agent of malaria. In addition, the inflammatory-type human group IIA (hGIIA) sPLA$_2$ circulates at high levels in the serum of malaria patients. However, the role of the different human sPLA$_2$s in host defense against *P. falciparum* has not been investigated. We show here that 4 out of 10 human sPLA$_2$s, namely, hGX, hGIIF, hGIII, and hGV, exhibit potent *in vitro* anti-*Plasmodium* properties with half-maximal inhibitory concentrations (IC_{50}s) of 2.9 ± 2.4, 10.7 ± 2.1, 16.5 ± 9.7, and 94.2 ± 41.9 nM, respectively. Other human sPLA2s, including hGIIA, are inactive. The inhibition is dependent on sPLA$_2$ catalytic activity and primarily due to hydrolysis of plasma lipoproteins from the parasite culture. Accordingly, purified lipoproteins that have been prehydrolyzed by hGX, hGIIF, hGIII, and hGV are more toxic to *P. falciparum* than native lipoproteins. However, the total enzymatic activities of human sPLA$_2$s on purified lipoproteins or plasma did not reflect their inhibitory activities on *P. falciparum*. For instance, hGIIF is 9-fold more toxic than hGV but releases a lower quantity of nonesterified fatty acids (NEFAs). Lipidomic analyses of released NEFAs from lipoproteins demonstrate that sPLA$_2$s with anti-*Plasmodium* properties are those that release polyunsaturated fatty acids (PUFAs), with hGIIF being the most selective enzyme. NEFAs purified from lipoproteins hydrolyzed by hGIIF were more potent at inhibiting *P. falciparum* than those from hGV, and PUFA-enriched liposomes hydrolyzed by sPLA$_2$s were highly toxic, demonstrating the critical role of PUFAs. The selectivity of sPLA$_2$s toward low- and high-density (LDL and HDL, respectively) lipoproteins and their ability to directly attack parasitized erythrocytes further explain their anti-*Plasmodium* activity. Together, our findings indicate that 4 human sPLA$_2$s are active against *P. falciparum in vitro* and pave the way to future investigations on their *in vivo* contribution in malaria pathophysiology.

Human malaria, a complex and deadly disease, is routinely caused by a protozoan parasite of the genus *Plasmodium* and transmitted by multiple species of the *Anopheles* mosquito. In 2012, the "Roll Back Malaria Report" made an estimate of 3.3 billion people (half of the world population) at risk of malaria, 219 million cases, and 660,000 deaths, most of them occurring in Africa and the Asia-Pacific (http://www.rollbackmalaria.org). The vast majority of clinical cases present as nonspecific febrile illnesses that are relatively easily terminated, but a minority of cases progress to a severe life-threatening disease. The major complications of severe malaria, including cerebral malaria and severe anemia, are almost exclusively due to *Plasmodium falciparum*. It is now commonly accepted that severe malaria is an extremely complex multiprocess and multisystem disorder (1). In the last 4 decades, the currently administered antimalarial treatments have become inefficacious because of the parasite resistance in most countries worldwide. The same risk of inefficacy is now starting to affect the effective ACT treatment that is based on artemisinin derivatives and related molecules (http://www.who.int/malaria/publications/world_malaria_report_2012/en/ and reference 2). In this context, new tools and strategies are urgently needed to fight against *Plasmodium*, as is a better understanding of the pathophysiological mechanisms of malaria.

We and others have shown that different snake and bee venom secreted phospholipases A_2 (sPLA$_2$s; EC 3.1.1.4) exhibit potent anti-*Plasmodium* properties *in vitro* (3–5). We demonstrated that venom sPLA$_2$s exert an indirect killing of *P. falciparum* through hydrolysis of human plasma phospholipids (PLs) present in the parasite culture medium (3, 4). We also demonstrated that the enzymatic hydrolysis of human lipoproteins by bee venom sPLA$_2$ generates lipid products that are toxic to the parasite (6). Nonesterified fatty acids (NEFAs), especially polyunsaturated NEFAs (PUFAs), were identified as the main mediators of parasite death.

sPLA$_2$s constitute a family of structurally conserved enzymes which are present in a broad range of living organisms, including plants, insects, and mammals (7, 8). All sPLA$_2$s are low-molecu-

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lar-mass proteins (14 to 19 kDa) that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release free fatty acids and lysophospholipids (lyso-PLs). Among them, human $sPLA_2$s form a family of up to 10 proteins referred to as groups IB, IIA, IID, IIE, IIF, III, V, X, XIIA, and XIIB, of which group XIIB $sPLA_2$ is catalytically inactive (9, 10). Human $sPLA_2$s exhibit different enzymatic properties (11) as well as unique tissue and cellular distributions (10), suggesting distinct physiological roles for each enzyme. Besides their role in lipid mediator production, accumulating evidence indicates that $sPLA_2$s participate in innate immunity, especially in the first line of host defense against bacteria and other pathogens (12–21).

Elevated levels of circulating $sPLA_2$ activity have been observed in the most severe cases of human malaria (22, 23). Based on immunological recognition and the absence of $sPLA_2$ activity in the parasite culture medium, the serum activity was attributed to human group IIA (hGIIA) $sPLA_2$. However, these studies were published in the early 1990s, when only human group IB (hGIB) and hGIIA $sPLA_2$s were known. Additionally, the possible antimalarial role of hGIIA $sPLA_2$ in response to infection by *P. falciparum* was not investigated.

We report here the anti-*Plasmodium* properties of the full set of human $sPLA_2$s in *in vitro* assays of *P. falciparum* development in human red blood cells (RBCs). In the presence of human plasma, recombinant human group IIF (hGIIF), III (hGIII), V (hGV), and X (hGX) $sPLA_2$s were toxic to *P. falciparum*, whereas all other $sPLA_2$s, including hGIIA, were inactive. Hydrolysis of lipoproteins rather than red blood cell membranes was found to be the main mechanism of $sPLA_2$ toxicity. However, the anti-*Plasmodium* activity of human $sPLA_2$s depends not on their overall hydrolytic activity on purified lipoproteins and plasma but rather on their specific ability to release PUFAs. Our results show for the first time the anti-*Plasmodium* activity of several human $sPLA_2$s and depict their mechanism of action. These findings will pave the way to future investigations on their possible contribution in malaria pathophysiology.

MATERIALS AND METHODS

Materials. Purified recombinant human $sPLA_2$s and the hGIII $sPLA_2$ domain were prepared as described previously (11, 24). The proenzyme form of hGX $sPLA_2$ (ProhGX) and the H48Q mutant of hGX $sPLA_2$ were produced as for mature wild-type (WT) hGX $sPLA_2$ using the pAB3 vector, in which the cDNA coding for the $sPLA_2$ was inserted in frame with the ΔGST protein and the factor Xa cleavage site, which were removed after cleavage by the factor Xa protease (11, 25). RPMI 1640 and Albumax II were from Life Technologies (Cergy Pontoise, France). Diff-Quik staining reagents were from Siemens Healthcare Diagnostics (Saint-Denis, France). The NEFA-C and the phospholipid (PL) B kits, used for the quantitative determination of nonesterified fatty acids (NEFAs) and PLs, respectively, were from Wako Chemicals (Oxoid S.A., Dardilly, France). Me-indoxam and the $sPLA_2$ inhibitor LY329722 [3-(3-aminooxalyl-1-benzyl-2-ethyl-6-methyl-1*H*-indol-4-yl)-propionic acid], which targets hGX $sPLA_2$, were synthesized as described previously (11, 26). High-quality biochemical reagents were from Sigma.

Culture and synchronization of *P. falciparum*. The Colombian strain FcB1 of *P. falciparum* was used throughout the work. Parasites were routinely grown at 37°C in human A^+ red blood cells (RBCs) at 2% hematocrit and 2 to 5% parasitemia in a 3% CO_2, 6% O_2, and 91% N_2 atmosphere. RPMI medium consisted of RPMI 1640 (Invitrogen, Inc.) supplemented with 11 mM glucose, 27.5 mM $NaHCO_3$, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin, adjusted to pH 7.4. To support parasite growth, RPMI medium was supplemented with 8% heat-inactivated human A^+ plasma (complete culture medium), according to the procedure of Trager and Jensen (27). When specified, Albumax II (0.5% final) was used in culture medium instead of heat-inactivated human plasma. Cultures were enriched in early stages of parasite development by sorbitol treatment (28).

Anti-*Plasmodium* activity assays with recombinant human $sPLA_2$s. Assays were performed according to the method of Desjardins et al. (29) and as reported previously (4). Briefly, lyophilized preparations of recombinant human $sPLA_2$s were dissolved at 50 μM in RPMI medium supplemented with 0.05% bovine serum albumin (BSA) and stored frozen until use. Decreasing concentrations of $sPLA_2$ in complete culture medium were distributed in 96-well microplates, and an FcB1 culture (1% final parasitemia, 2% final hematocrit) was added to the wells. Microplates were incubated for 24 h in a candle jar at 37°C before addition of radiolabeled [^{3}H]hypoxanthine (0.5 μCi/well). Parasite-incorporated radioactivity was measured 24 h later onto a fiberglass filter and counted in a 1450 Microbeta counter (Wallac, PerkinElmer). Percent growth inhibition was determined from the parasite-associated radioactivity measured in the presence and absence of $sPLA_2$. Half-maximal inhibitory concentrations (IC_{50}s) were determined from the $sPLA_2$ dose-response curves. Dose-response curves with $sPLA_2$ were performed with various batches of human plasma and red blood cells, leading to 3- to 5-fold variations of IC_{50}s (as exemplified in Fig. 1A and B for hGX $sPLA_2$, with mean IC_{50}s as indicated in Table 1). For dose-response assays in Albumax II, $sPLA_2$s were diluted in RPMI medium containing 0.5% Albumax II instead of heat-inactivated plasma and the FcB1 culture was grown for at least two parasite cycles in RPMI medium–0.5% Albumax II before use.

Inhibition of the anti-*Plasmodium* activities of hGX and hGV $sPLA_2$s by LY329722 and Me-indoxam. An FcB1 culture in complete medium was grown for 48 h in the presence of 7.5 nM recombinant hGX $sPLA_2$, with or without 20 μM LY329722, an inhibitor of hGX $sPLA_2$ (26). As controls, parasites were grown in the absence of hGX $sPLA_2$ and LY329722 and in the presence of LY329722 alone. Parasitemia (percentage of infected red blood cells in the culture) was determined from Diff-Quik-stained smears before and after incubation. Parasite development was assessed by multiplication factor (parasitemia at 48 h divided by initial parasitemia). For hGV $sPLA_2$, the enzyme was mixed at 500 nM in complete culture medium with 1.3 μM Me-indoxam, a high-affinity inhibitor of hGV $sPLA_2$ (11). Decreasing concentrations of the mix were then distributed in 96-well microplates to perform the dose-response assays as described above. As controls, parasites were grown in the presence of Me-indoxam or hGV added alone at the same respective concentrations.

Hydrolysis of human plasma by $sPLA_2$. Individual heat-inactivated plasma or mixtures of plasma from at least five different healthy donors were used in independent experiments. Plasma samples were incubated at 37°C with $sPLA_2$s at various concentrations. Plasma samples without $sPLA_2$ were incubated in parallel. Aliquots were taken at 0, 15, 30, 60, and 120 min and immediately frozen at −20°C. All samples were analyzed for NEFA content after thawing on ice, by using the NEFA-C kit according to the manufacturer's instructions. Values were normalized by subtracting NEFAs from controls without $sPLA_2$. Specific activities in micromoles of NEFA per minute per milligram of $sPLA_2$ were determined from the linear part of the kinetics curve [NEFA] = $f(t)$, with $f(t)$ being function of time.

Enzymatic assays on *Escherichia coli* membranes. Recombinant $sPLA_2$s were routinely checked for enzymatic activity through hydrolysis of *E. coli* membranes radiolabeled with [^{3}H]oleic acid and autoclaved (11). Briefly, 60 μl of radiolabeled *E. coli* membranes (100,000 dpm in activity buffer, i.e., 0.1 M Tris-HCl [pH 8.0], 10 mM $CaCl_2$, and 0.1% BSA) were incubated with $sPLA_2$ (0.01 to 1 nM final) at 37°C for various times (up to 1 h). Enzymatic reactions were stopped by adding 80 μl of 0.1 M EDTA–0.2% fatty acid-free BSA. Tubes were spun down for 5 min at 10,000 × *g*, and the supernatant was collected and counted in a 1450 Microbeta counter (Wallac, PerkinElmer). To test inhibition by Albumax II, radiolabeled *E. coli* membranes were resuspended in RPMI medium supplemented with 1 mM $CaCl_2$ or in RPMI-$CaCl_2$ containing 0.5% Al-

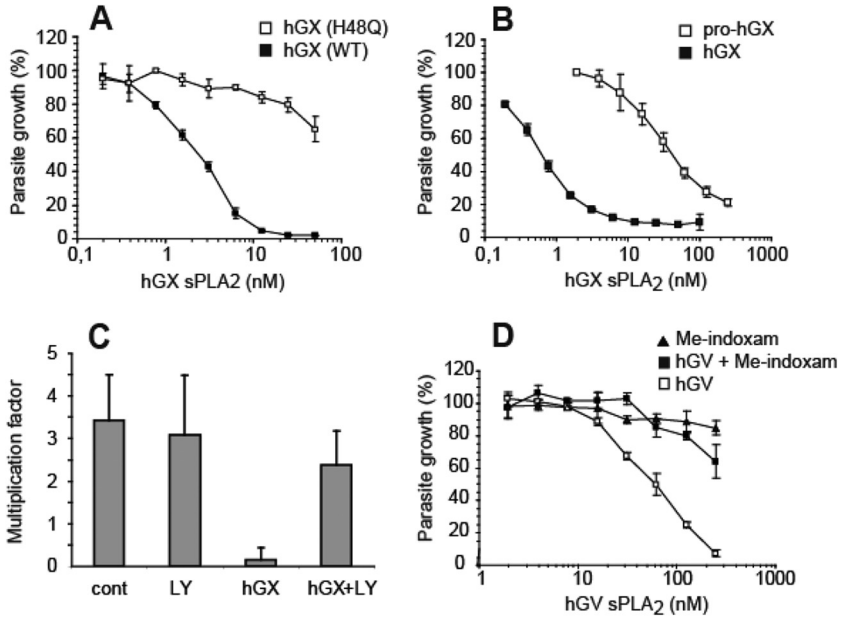

FIG 1 The anti-*Plasmodium* activity of hGV and hGX sPLA$_2$s requires their catalytic activity. (A and B) Mature WT hGX sPLA$_2$ (■) is much more active than its H48Q catalytically inactive mutant (□ in panel A) and its catalytically inactive proenzyme form (□ in panel B) at inhibiting the growth of *P. falciparum* strain FcB1. Parasite growth was measured by labeling of neosynthesized nucleic acids via [^{3}H]hypoxanthine utilization by *P. falciparum*. Growth was determined from control parasites incubated without sPLA$_2$. Values are means ± SDs of triplicate measurements. Note that dose-response assays whose results are shown in panels A and B were performed with different batches of human plasma and RBCs, leading to small differences in the IC$_{50}$s for hGX WT protein. (C) LY329722, a potent inhibitor of hGX catalytic activity, inhibits hGX anti-*Plasmodium* activity. An FcB1 culture was grown for 48 h in the presence of 7.5 nM recombinant hGX sPLA$_2$, with (hGX+LY) or without (hGX) 20 μM LY329722. Two negative controls were performed: parasites grown under normal culture conditions (cont) and in the presence of LY alone (LY). The multiplication factor was determined from Diff-Quik-stained smears before and after 48 h of incubation (see Materials and Methods). (D) Me-indoxam, a specific inhibitor of sPLA$_2$ catalytic activity, prevents hGV anti-*Plasmodium* activity. A dose-response assay for hGV anti-*Plasmodium* activity was performed in the presence (■) or absence (□) of Me-indoxam (see Materials and Methods). Control for Me-indoxam effect on *P. falciparum* was assessed by growing cells with Me-indoxam alone (▲). Growth was determined from parasites incubated without sPLA$_2$ or Me-indoxam. Values are means ± SDs of triplicate measurements.

bumax II instead of activity buffer, and sPLA$_2$s were added to a final concentration of 0.1 nM (hGV and hGX sPLA$_2$s), 0.3 nM (hGIII sPLA$_2$), or 0.4 nM (hGIIF sPLA$_2$).

Purification of lipoproteins. Low- and high-density lipoproteins (LDL and HDL, respectively) from nonfasted human plasma were prepared by differential centrifugation according to the procedure of Havel et al. (30). Briefly, chylomicrons and very-low-density lipoprotein (VLDL) were removed by a round of centrifugation at a density of 1.006 g/ml, and then LDL and HDL were either purified separately by successive centrifugations at densities of 1.053 g/ml and 1.210 g/ml, respectively, or copurified by a single run at a density of 1.210 g/ml. Lipoproteins were dialyzed at 4°C against NaCl (9 g/liter), then against RPMI medium alone, and then sterilized by filtration with a 0.2-μm membrane. They were stored at 4°C under a N_2 atmosphere and in the dark prior to experiments. Experiments were performed within 4 days of lipoprotein storage. Phospholipid content of lipoproteins (in terms of phosphatidylcholine [PC]) was measured by using the phospholipid B dosage kit from Wako Chemicals, according to the manufacturer's instructions.

TABLE 1 Anti-*Plasmodium* activities of human sPLA$_2$s

sPLA$_2$ (no. of expts)	IC$_{50}$ (nM)[a]
hGIB (2)	>1,250
hGIIA (5)	>1,250
hGIID (2)	>250
hGIIE (2)	>250
hGIIF (4)	10.7 ± 2.1
hGIII (3)	16.5 ± 9.7
hGV (4)	94.2 ± 41.9
hGX (6)	2.9 ± 2.4
hGXIIA (2)	>250
hGXIIB (2)	>250

[a] IC$_{50}$s are the means ± SDs of the indicated numbers of independent experiments.

Anti-*Plasmodium* assay in Albumax II medium supplemented with lipoproteins. A culture of *P. falciparum* in Albumax II (0.5% parasitemia and 2% hematocrit) was supplemented with copurified LDL and HDL (final concentration, 0.2 mg of PLs/ml) and incubated for 48 h under culture conditions with hGIIF, hGIII, hGV, and hGX sPLA$_2$s at respective concentrations of 50, 45, 200, and 10 nM. Controls were without sPLA$_2$ and/or without lipoproteins. Parasitemia was determined from Diff-Quik-stained smears before and after incubation. Multiplication rate was calculated as parasitemia after 48 h divided by the original parasitemia.

Lipoprotein hydrolysis by sPLA$_2$s. LDL and HDL were adjusted to 1 mg of PLs/ml in RPMI medium supplemented with 1 mM $CaCl_2$. Lipoproteins were incubated with and without sPLA$_2$ for various times at 37°C. sPLA$_2$s were used at different final concentrations: hGIB at 100 nM (number of independent experiments [n] = 4), hGIIA at 50, 100, or 200 nM (n = 5), hGIID at 100 or 200 nM (n = 2), hGIIE at 200 or 250 nM (n = 2), hGIIF at 30 or 40 nM (n = 5), hGIII at 50 or 100 nM (n = 4), hGV at 10, 40, or 50 nM (n = 6), hGX at 5, 10, or 20 nM (n = 6), and hGXIIA at 100 or 200 nM (n = 2). NEFAs were measured at different time points using the NEFA-C kit (Wako) according to the manufacturer's instructions. Values were corrected by subtracting NEFAs measured for lipoproteins incubated without sPLA$_2$. Specific activities were determined from the linear part of the curve [NEFA] = $f(t)$.

Anti-*Plasmodium* activity of sPLA$_2$-hydrolyzed lipoproteins. The ability of human sPLA$_2$s to promote the toxicity of lipoproteins was tested

in dose-response assays. Two hundred microliters of LDL and HDL (0.6 mg of PLs/ml in RPMI medium) was incubated overnight at 37°C with 20 nM $sPLA_2$ or alone. Decreasing concentrations of each incubated volume were then distributed in a 96-well microtiter plate and mixed with an equal volume of FcB1 culture in RPMI–1.0% Albumax II for dose-response assays. Albumax II instead of human plasma was used throughout the test to avoid any contribution of lipoproteins from human plasma. Incubations with each $sPLA_2$ added alone were also performed to check for non-lipoprotein-dependent toxicity of $sPLA_2$s under these conditions.

Lipidomic analyses of $sPLA_2$-hydrolyzed lipoproteins. LDL and HDL were purified from a mixture of 12 human normal plasma samples, dialyzed against 0.1 M Tris-HCl (pH 7.4), and then diluted in the same buffer to 0.85 and 1.0 mg of PC/ml for LDL and HDL, respectively. Hydrolysis of lipoproteins was performed at 37°C in the presence of 0.01% fatty acid-free BSA and 1 mM $CaCl_2$, with recombinant $sPLA_2$s added at the following final concentrations: hGIIF, 75 nM (LDL) and 30 nM (HDL); hGIII, 45 nM (LDL) and 100 nM (HDL); hGV, 50 nM (LDL) and 13 nM (HDL); and hGX, 20 nM (LDL) and 6.5 nM (HDL). Enzyme concentrations were chosen according to the specific activities of $sPLA_2$s on LDL and HDL, to hydrolyze less than 10% PC after 4 h of incubation. After incubations (1, 4, and 18 h), tubes were put on ice and 2 volumes of ice-cold methanol were added. Tubes were vortexed, shortly flushed with nitrogen, and frozen at −80°C. Parallel incubations were made with lipoproteins alone. Lipidomic analyses, i.e., sample extractions for lysophospholipids and NEFAs, derivatization of NEFAs, and quantification by using liquid chromatography-tandem mass spectrometry (LC-MS/MS), were performed as described previously (31, 32).

Anti-*Plasmodium* activity of liposomes enriched with PUFAs after hydrolysis by $sPLA_2$s. We prepared liposomes with and without PUFAs of the following phospholipid compositions: liposomes without PUFAs, 50% PC (16:0; 18:1)–30% phosphatidylethanolamine (PE) (16:0; 18:1)–20% phosphatidylserine (PS) (16:0; 18:1); liposomes with PUFAs, 25% PC (18:0; 20:4)–25% PC (18:0; 22:6)–15% PE (18:0; 20:4)–15% PE (18:0; 22:6)–20% PS (16:0; 18:1) (all phospholipids were from Avanti Polar Lipids). The two lipid films were made in a rotary evaporator and resuspended in 1 ml of 50 mM HEPES (pH 7.2)–120 mM potassium acetate (the buffer was freshly degassed to prevent oxidation). The liposome suspensions were then frozen and thawed 5 times to reduce multilamellarity, followed by 19 extrusion cycles through a 0.4-μm polycarbonate filter using an Avanti extruder. The liposomes were resuspended in 1 ml (final volume) of buffer at 1 mg of PLs/ml and stored frozen at −20°C under argon until use.

The capacity of the two liposomal preparations to mediate toxicity of hGIIF, hGIII, hGV, and hGX $sPLA_2$s against *P. falciparum* was analyzed as follows. Aliquots (100 μl) of a parasite culture (0.5% parasitemia and 2% hematocrit) in RPMI–0.5% Albumax II were distributed in a 96-well microplate. Liposomes (final concentration of 65 μg of PC/ml) and hGIIF (50 nM), hGX (10 nM), hGV (200 nM), and hGIII (45 nM) $sPLA_2$s were then added. The microplate was incubated for 48 h in a candle jar at 37°C. Control wells without liposomes and/or without $sPLA_2$s were run on the same plate. Parasite multiplication was established by determining parasitemia from Diff-Quik-stained smears before and after the 48-h incubation period.

Anti-*Plasmodium* activity of NEFAs extracted from $sPLA_2$-hydrolyzed lipoproteins. A mixture of 5 human normal plasma samples was diluted with 2 volumes of NaCl (9 g/liter) and centrifuged for 24 h at 120,000 × *g* to remove VLDL and chylomicrons (30). A combined fraction of both LDL and HDL was purified by centrifugation (120,000 × *g*, 24 h, 4°C) of the plasma adjusted to a density of 1.21 g/cm^3 with KBr and then extensively dialyzed at 4°C against 0.1 M Tris-HCl (pH 7.4). The LDL-HDL fraction was supplemented with 1 mM $CaCl_2$ and filtered with a 0.2-μm membrane, and 1-ml samples were incubated at 37°C for 18 h with hGV (50 nM) or hGIIF (60 nM) $sPLA_2$s or no enzyme. After incubation, 5 volumes of 2% (wt/vol) fatty acid-free BSA in NaCl (9 g/liter) were added, and samples were rotated for 45 min at room temperature and then centrifuged at 120,000 × *g* for 24 h at 4°C in the presence of KBr (1.21 g/cm^3). The pelleted BSA was extensively dialyzed at 4°C against phosphate-buffered saline (PBS). NEFAs were extracted from BSA using Dole's procedure (33). Extracted NEFAs were dried under nitrogen, resuspended in 0.2 ml of RPMI medim–8% heat-inactivated plasma, and stored frozen at −80°C. Purity of the NEFA fractions was assessed by measuring the content of NEFAs and PLs using the NEFA-C and phospholipid B kits, respectively. Samples were found to be essentially free of PLs (data not shown). NEFAs were assayed for parasite growth inhibition by dose-response assays.

Membranolytic activity of human $sPLA_2$s on *Plasmodium*-infected RBCs. For $sPLA_2$ treatment of infected RBCs, a parasite culture was enriched in young parasite stages (rings to early trophozoites, i.e., 0 to 20 h postinvasion) by sorbitol treatment. Parasitemia (3 to 3.5%) and stage distribution were determined by optical examination of Diff-Quik-stained smears by cell counting and morphological examination of the parasites, respectively. One half of the culture was processed immediately, whereas the other half was maintained under normal culture conditions for 24 h to develop until the schizont stage. Noninfected RBCs were maintained under culture conditions for 24 h prior to treatment. Parasite cultures and healthy RBCs were processed as follows. Cells were pelleted for 2 min at 900 × *g*, and then 1 volume (100 μl) of packed RBCs was washed three times in 100 volumes of RPMI medium and resuspended in 1 volume of RPMI medium–0.05% BSA. Fifty microliters of the cell suspension (~12.5 μg of PLs) was distributed in a 96-well microplate. Small volumes (up to 2 μl) of recombinant $sPLA_2$s (hGIB, hGIIA, hGIIF, hGIII, hGV, and hGX) in PBS–0.02% BSA were added at final concentrations of 100, 250, 50, 50, 50, and 30 nM, respectively. Human plasma diluted in RPMI medium was distributed into wells at the same PL concentration (12.5 μg of PLs/well) and was incubated with the enzymes under the same conditions to compare $sPLA_2$ activities on cells and plasma. Controls for the spontaneous production of NEFAs by RBCs and plasma were performed without enzyme. The microplate was incubated in a candle jar for 5 h at 37°C. Plasma samples and RBC supernatants were taken and frozen at −20°C. Ghosts were prepared from RBCs by hypotonic lysis in ice-cold 5P8 as described below and frozen at −20°C. Samples were thawed on ice just before measurement of NEFAs in triplicate using the NEFA-C kit. NEFAs in RBC samples were expressed as the sum of NEFAs released from ghosts and the respective supernatants.

Preparation of RBC ghosts. Erythrocyte ghosts were prepared by following the procedure of Dodge et al. (34), with minor modifications. One hundred microliters of packed human red blood cells (~10^9 RBCs) was washed in PBS at room temperature, then lysed in 10 volumes of ice-cold 5P8 (5 mM sodium phosphate, pH 8.0), and centrifuged at 14,000 × *g* for 15 min at 4°C. Pelleted ghosts were washed several times in ice-cold 5P8 to fully remove hemoglobin and resuspended with 5P8 up to the initial volume (100 μl). PL content in ghosts was measured by using the phospholipid B kit. It was estimated at 0.5 g/liter (volume of packed RBCs) from 4 independent measurements (RBCs from different donors).

Statistical analysis. Data were analyzed using GraphPad InStat 3 software (San Diego, CA). Normality was tested using the Shapiro-Wilk test for groups for which the number was superior to 6. When the number was too small (≤6), the distribution was considered nonparametric. For experiments with purified lipoproteins, values resulting from a given LDL and HDL treatment were considered to be matched, since LDL and HDL were both purified from the same plasma or mix of plasma samples and handled in parallel. When sampling distribution was found to be normal, parametric paired *t* test with a two-tailed *P* value was applied. When sampling distribution was not normal and/or the number was too small, the nonparametric Wilcoxon matched-pair (signed rank) test was used. For experiments with liposomes ($n = 5$), Friedman test (repeated measures test, nonparametric) with Dunn's multiple-comparison posttest was applied. A *P* value of <0.05 was considered significant, and a *P* value of <0.001 was considered extremely significant.

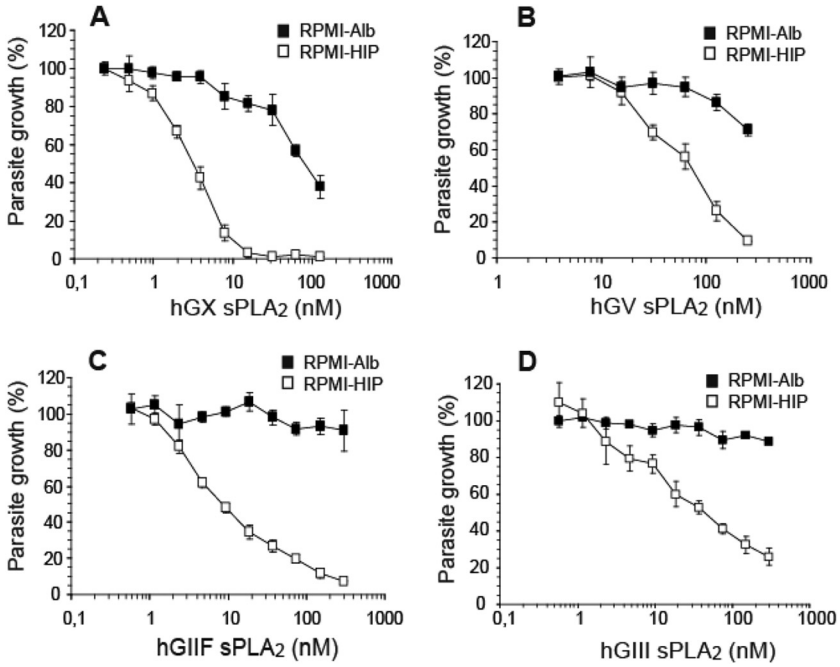

FIG 2 Effect of plasma depletion on the anti-*Plasmodium* activity of human sPLA$_2$s. Dose-response assays with recombinant hGX (A), hGV (B), hGIIF (C), and hGIII (D) sPLA$_2$s were performed in RPMI medium supplemented with 0.5% (wt/vol) Albumax II (RPMI-Alb) or under normal culture conditions, i.e., RPMI supplemented with 8% (vol/vol) human heat-inactivated plasma (RPMI-HIP). Values are means ± SDs of triplicate measurements.

RESULTS

Human group IIF, III, V, and X sPLA$_2$s inhibit the development of *P. falciparum* in human RBCs. The full set of recombinant human sPLA$_2$s (groups IB, IIA, IID, IIE, IIF, III, V, X, XIIA, and XIIB) was tested in dose-response assays for growth inhibition of *P. falciparum* in the presence of human normal plasma. Three sPLA$_2$s, i.e., hGIIF, hGIII, and hGX, were highly inhibitory. hGX sPLA$_2$ was the most active, with an average IC$_{50}$ of 2.9 ± 2.4 nM (Table 1 and Fig. 1). hGIIF and hGIII exhibited IC$_{50}$s of 10.7 ± 2.1 nM and 16.5 ± 9.7 nM, respectively. hGV sPLA$_2$ was also inhibitory, but only when added at high concentrations (IC$_{50}$ of 94.2 ± 41.9 nM). In contrast, hGIIA and other human sPLA$_2$s were inactive at concentrations as high as 250 to 1,250 nM (Table 1).

The catalytic activity of human sPLA$_2$s is required for their anti-*Plasmodium* effect. The role of hGX catalytic activity in parasite inhibition was analyzed using three specific tools: the H48Q active-site mutant of the enzyme, which has less than 0.1% of WT catalytic activity (35); the catalytically inactive proenzyme form of hGX, which has less than 0.2% of WT activity (25), and LY329722, a potent active-site inhibitor of hGX (26). In dose-response assays, the H48Q mutant was much less active than the WT enzyme against *P. falciparum*, inducing only a modest inhibition of growth at 50 nM (Fig. 1A). The recombinant proenzyme form of hGX was also poorly inhibitory, exhibiting a 65-fold-higher IC$_{50}$ than that of WT enzyme (Fig. 1B). Finally, incubation of hGX with LY329722 fully restored the parasite growth (Fig. 1C). Together, these results clearly demonstrate that the catalytic activity of hGX is crucial for its parasiticidal activity. To evaluate the role of catalytic activity in the anti-*Plasmodium* effect of hGV, we used Me-indoxam, currently known as the most potent inhibitor of this sPLA$_2$ (11). As shown in Fig. 1D, Me-indoxam prevented the effect of hGV, indicating that the anti-*Plasmodium* effect of hGV also relies on its enzymatic activity. Since LY329722 and Me-indoxam are only weak inhibitors of hGIIF and hGIII sPLA$_2$s (11, 36), we did not evaluate the role of enzymatic activity in their anti-*Plasmodium* effect with these inhibitors.

Hydrolysis of exogenous phospholipids from plasma is the prominent mechanism of sPLA$_2$ toxicity. The *in vitro* anti-*Plasmodium* activity of bee venom sPLA$_2$ was found to be mediated by hydrolysis of PLs present in the culture medium, more specifically, from human plasma lipoproteins, thereby generating some NEFAs that are directly toxic to the parasite (6). A similar mechanism by hydrolysis of exogenous PLs might be involved in the anti-*Plasmodium* activity of human sPLA$_2$s. We thus evaluated the respective contribution of plasma hydrolysis versus red blood cell hydrolysis in the toxicity of the anti-*Plasmodium* human sPLA$_2$s by performing dose-response assays of sPLA$_2$ under conditions where the human plasma was replaced by Albumax II, a lipid-rich bovine albumin preparation free of phospholipids and lipoproteins (4). As shown in Fig. 2, replacement of human plasma with Albumax II dramatically reduced the anti-*Plasmodium* activity of all 4 sPLA$_2$s hGX, hGV, hGIII, and hGIIF. A 25-fold shift in the IC$_{50}$ of hGX sPLA$_2$ was observed when human plasma was replaced with Albumax II (Fig. 2A). Similarly, 250 nM hGV sPLA$_2$ in Albumax II induced only a 30% parasite inhibition, while the same concentration induced a 90% inhibition in plasma (Fig. 2B). Finally, hGIIF and hGIII sPLA$_2$s were fully inactive in Albumax II at concentrations as high as 300 nM (Fig. 2C and D, respectively).

To confirm that the altered toxicity of hGIIF, hGIII, hGV, and hGX sPLA$_2$s in Albumax II results from the absence of exogenous PL substrate (lipoproteins) and is not due to an impairment of enzymatic activity by the lipid-rich albumin, we analyzed the effect of Albumax II on the enzymatic activity of sPLA$_2$s using radiolabeled *E. coli* membranes, a well-known and highly sensitive

$sPLA_2$ substrate (11). As shown in Fig. S1 in the supplemental material, hydrolysis of *E. coli* membranes by hGIIF and hGX was lowered approximately 2-fold, whereas that of hGIII and hGV was not modified by the presence of Albumax II. These results indicate that Albumax II cannot explain on its own the drop in toxicities of hGIII and hGV $sPLA_2$s in the absence of plasma. Likewise, the total loss of anti-*Plasmodium* toxicity of hGIIF $sPLA_2$ under the same conditions could not result from its only partial impairment by Albumax II. Last, the 25-fold drop in IC_{50} of hGX is most probably not explained by the 2-fold alteration of its enzymatic activity by Albumax II. Furthermore, addition of purified lipoproteins in the presence of Albumax II fully restored the anti-*Plasmodium* activity of $sPLA_2$s (see below).

Overall, these results indicate that the anti-*Plasmodium* activity of all four $sPLA_2$s depends to a large extent on the presence of exogenous PLs from plasma, yet hGX and hGV retained a moderate anti-*Plasmodium* activity in the absence of plasma.

The membranolytic activity of hGV and hGX $sPLA_2$s on human RBCs infected by *P. falciparum* contributes marginally to their anti-*Plasmodium* activity. In addition to the above-described role of human plasma in the $sPLA_2$ anti-*Plasmodium* activity, the direct hydrolysis of RBCs by $sPLA_2$ may also contribute to parasite inhibition. In particular, this would be the case for hGX and hGV, which retained some inhibitory activity in the absence of plasma at high concentrations (Fig. 2). This possibility is also likely since the plasma membrane of various mammalian cells, either in a resting state or during apoptosis, is susceptible to membrane attack by several, but not all, human $sPLA_2$s (37). Among the latter, hGX appears to be the most active enzyme against normal and damaged cells. On the other hand, hGIIA was tested on human RBCs and found to attack PS-exposing RBCs but not normal RBCs (38).

We thus investigated whether the anti-*Plasmodium* activity of hGX, hGIIF, hGIII, and hGV $sPLA_2$s might also rely on their propensity to attack membranes of *Plasmodium*-infected human RBCs. Furthermore, since the host RBC membrane undergoes major phospholipid reorganizations during the different stages of parasite maturation (39) and since such changes are likely to modify the interfacial binding properties of $sPLA_2$s and their capacity to release lysophospholipids and fatty acids (40), we analyzed the release of NEFAs by $sPLA_2$s when incubated with parasite cultures enriched in either young (early trophozoites) or mature (late trophozoites/schizonts) parasites. For this experiment, the cell-hydrolyzing activities of 4 toxic (hGX, hGIIF, hGIII, and hGV) and 2 nontoxic (hGIB and hGIIA) $sPLA_2$s against *P. falciparum* were compared. The specific activities of $sPLA_2$s on healthy and infected red blood cells were calculated from independent experiments with RBCs from different donors and are presented in Fig. 3. hGX $sPLA_2$ readily hydrolyzed RBCs and appeared to prefer infected RBCs, especially those infected by the mature forms of *P. falciparum*. However, the enzyme was about 3-fold less active on cultures enriched with mature parasites than on plasma. hGV $sPLA_2$ also exhibited substantial, although lower, hydrolysis of infected erythrocytes. Similar to the case with hGX, hydrolysis of plasma by hGV was higher than cell hydrolysis, at least 5-fold. Very modest hydrolysis of RBCs, infected or not, was observed with the other $sPLA_2$s, regardless of their capacity to hydrolyze plasma. Thus, in general, anti-*Plasmodium* $sPLA_2$s hydrolyze more potently plasma than RBC plasma membrane, infected or not. In addition, of all the $sPLA_2$s, hGX and hGV $sPLA_2$s exhibit

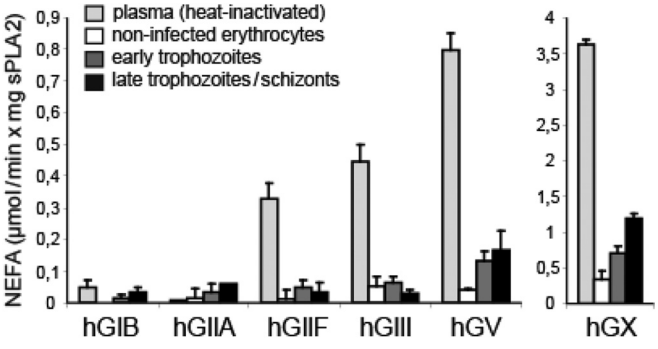

FIG 3 Membranolytic activity of human $sPLA_2$s on *P. falciparum*-infected RBCs. A semisynchronous culture of the FcB1 strain of *P. falciparum* (3.5% parasitemia) was analyzed for its sensitivity to the cell membrane-hydrolyzing activity of hGIB, hGIIA, hGIIF, hGIII, hGV, and hGX $sPLA_2$s at early and late development stages. Parasitemia was determined by counting 2,000 RBCs on Diff-Quik-stained smears. Pelleted RBCs from the culture at time zero (early trophozoites) and 24 h (late trophozoites/schizonts) were resuspended in RPMI medium supplemented with 0.05% BSA and incubated for 5 h at 37°C in a candle jar with 100 nM hGIB, 250 nM hGIIA, 50 nM hGIIF, 50 nM hGIII, 50 nM hGV, and 30 nM hGX $sPLA_2$. Noninfected RBCs were processed similarly. Human heat-inactivated plasma (plasma) was incubated under the same conditions. After incubation, NEFAs were measured using the NEFA-C kit (Wako) by following the manufacturer's instructions, with oleic acid as the standard. Values are means ± SDs from triplicate determination.

the most potent activities on infected red blood cells. For these two $sPLA_2$s, the anti-*Plasmodium* effects might thus be explained to a large degree by their potent hydrolytic activity on plasma phospholipids and to a lesser degree by their ability to hydrolyze RBC membranes. The latter activity would explain the remaining anti-*Plasmodium* activity of hGX and hGV $sPLA_2$s in the absence of plasma but in the presence of infected RBCs (Fig. 2).

Plasma lipoproteins play a major role in the anti-*Plasmodium* activity of human $sPLA_2$s. The above-described findings suggested that hydrolysis of plasma phospholipids is the central mechanism of anti-*Plasmodium* activity of human $sPLA_2$s. Since lipoproteins constitute the major source of PLs in human plasma, while bee venom $sPLA_2$ is toxic to *Plasmodium* by hydrolysis of VLDL (6), we hypothesized that lipoproteins may play a major role in $sPLA_2$ toxicity. We tested this hypothesis by incubating hGIIF, hGIII, hGV, and hGX $sPLA_2$s with a parasite culture in the presence of Albumax II, i.e., under a condition in which the enzymes are not toxic by themselves, and trying to restore the anti-*Plasmodium* activities of $sPLA_2$s by addition of purified lipoproteins (Fig. 4). As expected, parasites exhibited normal growth when incubated with hGIIF, hGIII, hGV, or hGX $sPLA_2$s in Albumax II alone, whereas addition of lipoproteins led to a marked inhibition of the parasite development with each $sPLA_2$. These data clearly demonstrated that lipoproteins constitute the preferential substrate of the 4 human $sPLA_2$s compared to red blood cells and play a major role in $sPLA_2$ toxicity.

The global enzymatic activities of human $sPLA_2$s on plasma and purified lipoproteins only partially correlate with their anti-*Plasmodium* activities. Since several human $sPLA_2$s efficiently hydrolyze purified lipoproteins (41–44), we measured their enzymatic activities on both total plasma and lipoproteins (Table 2). Interestingly, the $sPLA_2$ enzymatic activities have been investigated in serum but not in plasma, even though plasma is a complex environment that might modify the interactions between

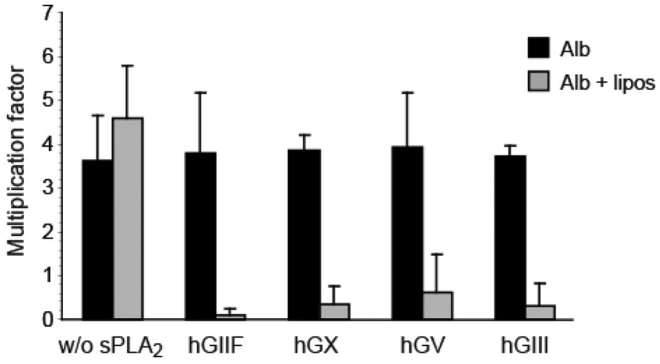

FIG 4 Ability of lipoproteins to induce anti-*Plasmodium* activity of human sPLA$_2$s in the absence of plasma. A culture of *P. falciparum* in RPMI–0.5% Albumax II was distributed in a 96-well microplate and incubated with hGIIF (50 nM), hGX (10 nM), hGV (200 nM), and hGIII (45 nM) sPLA$_2$s or without sPLA$_2$ (w/o sPLA$_2$). The culture medium was either Albumax II alone (Alb) or Albumax II supplemented with copurified LDL and HDL (Alb+Lipos). Parasite development was determined from Diff-Quik-stained smears established at time zero of incubation and after 48 h of incubation.

sPLA$_2$s and lipoproteins. To first determine the respective efficiencies of human sPLA$_2$s at hydrolyzing total plasma, the enzymes were incubated with heat-inactivated human plasma as used for the parasite culture, and accumulation of NEFAs was measured. The rank order of hydrolytic potency in total plasma was hGX > hGV > hGIII > hGIIF ≫ hGIB and hGIIA (Table 2). hGX sPLA$_2$ was the most active in releasing NEFAs, with a specific activity of 2.99 ± 1.91 μmol of NEFA/min/mg of enzyme, whereas hGIIF sPLA$_2$ was the least active among anti-*Plasmodium* sPLA$_2$s, with a specific activity of 0.28 ± 0.11 μmol of NEFA/min/mg of enzyme (Table 2). The activity of hGIIA was barely detectable (<0.01 μmol of NEFA/min/mg of enzyme). No activity could be measured under our experimental conditions for other human sPLA$_2$s (hGIID, hGIIE, and hGXIIA [data not shown]).

We next measured the enzymatic activities of the different human sPLA$_2$s on purified LDL and HDL lipoproteins by measuring the release of total NEFAs (Table 2). hGX, hGV, hGIII, and hGIIF sPLA$_2$s were the most active enzymes on both LDL and HDL, but with various efficacies. The rank orders of sPLA$_2$ hydrolytic activity on LDL and HDL were hGIII > hGX > hGV > hGIIF and hGX > hGV > hGIIF > hGIII, respectively. hGIB sPLA$_2$ also degraded lipoproteins at a low rate and appeared to prefer HDL. The other sPLA$_2$s were barely active and were virtually inactive in the case of hGXIIA. The enzymatic activities of sPLA$_2$s on total plasma were in accordance with those measured on purified lipoproteins (Table 2 and references 41 to 44), indicating that heat-inactivated plasma components do not appreciably interfere with the hydrolysis of lipoproteins by sPLA$_2$s. They also show that hGX, hGV, and hGIIF have a significant preference for HDL, as may have hGIB, while hGIII sPLA$_2$ appears to be more active on LDL (Table 2), as previously noted by Sato et al. (44).

TABLE 2 Specific activities of human sPLA$_2$s on plasma and purified lipoproteins[a]

sPLA$_2$	Sp act (μmol of NEFA/min/mg sPLA$_2$)			*P* value (LDL vs HDL)
	Plasma	LDL	HDL	
hGIB	0.04 ± 0.02 (4)	0.05 ± 0.02 (4)	0.25 ± 0.22 (4)	0.06
hGIIA	<0.01 (6)	0.02 ± 0.02 (7)	0.03 ± 0.03 (7)	0.56
hGIID	ND	0.03 (2)	0.04 (2)	ND
hGIIE	ND	0.02 (2)	0.02 (2)	ND
hGIIF	0.28 ± 0.11 (10)	0.74 ± 0.21 (6)	1.58 ± 0.59 (6)	0.03*
hGIII	0.44 ± 0.42 (5)	6.29 ± 5.37 (5)	1.06 ± 0.70 (5)	0.06
hGV	1.07 ± 0.94 (6)	1.05 ± 0.44 (6)	3.88 ± 2.20 (6)	0.03*
hGX	2.99 ± 1.91 (10)	2.58 ± 1.60 (6)	7.92 ± 5.08 (6)	0.01*
hGXIIA	ND	<0.01 (2)	<0.01 (2)	ND

[a] Values are means ± SDs from independent experiments. The number of experiments is given in parentheses. *P* values were determined from paired *t* test or Wilcoxon signed-rank test according to normal or nonnormal sampling distribution, respectively, with a *P* value of <0.05 considered significant (asterisk). ND, not determined.

TABLE 3 Anti-*Plasmodium* activities of sPLA$_2$-hydrolyzed lipoproteins[a]

sPLA$_2$	Anti-*Plasmodium* activity (IC_{50}, in μg of PC/ml)		*P* value (LDL vs HDL)
	LDL	HDL	
None	>250	>250	ND
hGIB	>250	>250	ND
hGIIA	>250	>250	ND
hGIID	226.6 ± 40.7	>250	ND
hGIIE	>250	238.7 ± 43.3	ND
hGIIF	190.0 ± 89.5	157.4 ± 68.9	0.06
hGIII	115.0 ± 43.9	210.2 ± 99.9	0.06
hGV	241.6 ± 133.8	181.2 ± 84.5	0.40
hGX	111.6 ± 37.3	81.8 ± 13.3	0.62
hGXIIA	>250	≥250	ND

[a] Values are means ± SDs from 5 independent experiments. *P* values were determined from Wilcoxon signed-rank test, with a *P* value of <0.05 considered significant. ND, not determined.

Of direct relevance to this study, only the 4 sPLA$_2$s (hGIIF, hGIII, hGV, and hGX) which were able to hydrolyze both total plasma and purified lipoproteins were those displaying anti-*Plasmodium* activity. However, hGIIF was rather modestly active on plasma, while it had a marked activity on *Plasmodium*. Conversely, hGV and hGIII displayed relatively strong activities in plasma, in between those of hGIIF and hGX, but were less potent than these enzymes on *Plasmodium*. Together, these results suggested that the anti-*Plasmodium* activity of the different human sPLA$_2$s requires the hydrolysis of plasma lipoproteins but does not solely depend on their global hydrolytic activities on lipoproteins.

hGIIF, hGIII, hGV, and hGX are the most active human sPLA$_2$s at generating toxic LDL and HDL. To further establish the link between the efficiency of sPLA$_2$s at inhibiting parasite development and their ability to hydrolyze lipoproteins, we analyzed the respective capacities of the human enzymes to generate toxic lipoprotein particles. A fixed concentration of LDL and HDL was pretreated for 15 h with a low concentration of each sPLA$_2$ (20 nM), after which the mixture was diluted and added to a parasite culture in Albumax II for dose-response assays. Under these conditions, addition of sPLA$_2$s alone failed to inhibit the parasite growth (data not shown). As shown in Table 3, hGIIF, hGIII, hGV, and hGX sPLA$_2$s converted LDLs and HDLs into particles toxic toward *Plasmodium*, but with distinct efficiencies. LDLs hydrolyzed by hGX and hGIII were most inhibitory, in good accordance with the highest efficiency of these sPLA$_2$s at hydrolyzing these particles. hGIIF-hydrolyzed LDLs were more active on *Plasmodium* than the hGV-hydrolyzed

ones (Table 3), although hGV was more active than hGIIF on LDL (Table 2). In short, the rank order toxicity for $sPLA_2$-hydrolyzed LDLs was hGX > hGIII > hGIIF > hGV. Considering HDLs, hGX-hydrolyzed HDLs were the most toxic, as expected from the high activity of hGX on these particles, whereas hGIII-hydrolyzed HDLs were weakly toxic, in accordance with the low activity of this enzyme on this class of lipoprotein. Again, hGIIF exhibited higher potency than hGV at generating toxic particles, in contrast with the respective activities of these enzymes on HDL. The rank order for $sPLA_2$-hydrolyzed HDLs was hGX > hGIIF > hGV > hGIII. Considering all these results together, it appears that $sPLA_2$-hydrolyzed lipoproteins exhibit anti-*Plasmodium* activities reflecting those of $sPLA_2$s under normal plasma-rich culture conditions, especially when considering the relative effects of hGIIF and hGV $sPLA_2$s, with the former being more inhibitory than the latter, and despite weaker enzymatic activity on purified lipoproteins and plasma.

Identification of free fatty acids released from LDL and HDL by the selective enzymatic activities of hGIIF, hGIII, hGV, and hGX $sPLA_2$s. The above-described data clearly indicate that the anti-*Plasmodium* activity of the different human $sPLA_2$s requires hydrolysis of phospholipids from lipoproteins but does not fully correlate with their global activity, suggesting the involvement of discrete enzymatic properties. Since it was shown that free PUFAs, especially arachidonic acid (AA), are more toxic to *Plasmodium* than free monounsaturated and saturated FAs (6, 45), while we further showed that bee venom $sPLA_2$-hydrolyzed VLDLs are inhibitory to *P. falciparum* due to toxic polyunsaturated NEFAs, we sought to determine whether the human $sPLA_2$s with anti-*Plasmodium* activity can release specific PUFAs. This hypothesis was also supported by studies showing that human $sPLA_2$s exhibit different selectivities toward lipoprotein-PC species with different fatty acids at the *sn*-2 position. Indeed, it was shown that hGX $sPLA_2$ preferentially hydrolyzes PC with linoleic acid (LA; 18:2) and arachidonic acid (20:4) at the *sn*-2 position, whereas hGV $sPLA_2$ prefers PC with LA and oleic acid (OA; 18:1) (41, 44, 46, 47). Furthermore, hGIII exhibited no preference toward PC species from LDL and HDL, whereas hGIIF $sPLA_2$ attacks PC species with AA preferentially (44).

The respective amounts of NEFAs and lysophospholipids released from LDL and HDL by hGIIF, hGIII, hGV, and hGX $sPLA_2$s were determined by lipidomic analyses using LC-electrospray ionization (ESI)-MS (Fig. 5; see also Table S1 in the supplemental material). As indicated by the vast majority of lyso-PC produced (data not shown), hGIIF, hGIII, hGV, and hGX $sPLA_2$s degraded much more PC than any other phospholipids from both LDL and HDL. This reflects the distribution of phospholipid classes in normal lipoproteins and is in accordance with previously published data (41, 48, 49). Lyso-PC species in hydrolyzed LDL and HDL were as follows: 16:0 > 18:0 > 18:1 > 18:2 for all four $sPLA_2$s (data not shown). Other lyso-PL species released by $sPLA_2$s included mainly lyso-PE, but in a much smaller amount than that of lyso-PC.

As expected from the specific activities of these $sPLA_2$s on LDL and HDL, hGIIF, hGV, and hGX $sPLA_2$s released larger amounts of NEFAs from HDL than LDL, whereas hGIII released more NEFAs from LDL. Specificities of hGIIF, hGIII, and hGX $sPLA_2$s for FAs at the *sn*-2 position of phospholipids in LDL and HDL were highly comparable, with the most produced FAs in the following order: 18:2 > 18:1 > 20:3 > 20:4 > 16:0≈18:0 (Fig. 4; see also Table S1 in the supplemental material). Interestingly, hGIIF was the most selective $sPLA_2$ at releasing AA and other PUFAs (see Table S1). Conversely, hGV generated almost no AA from both LDL and HDL even after 18 h of incubation, leading to this specific profile of NEFAs: 18:2 > 18:1 > 20:3 > 16:0≈18:0. All four $sPLA_2$s substantially released dihomo-gamma-linolenic acid (DGLA; 20:3), which was rather unexpected since DGLA is usually at lower concentrations than AA in normal lipoproteins. This might result from a specific enrichment in DGLA while mixing different human plasma samples, but in any case, it indicates a selective action of some human $sPLA_2$s toward DGLA. In line with a previous publication by Gesquiere et al. (50), hGV $sPLA_2$ did release DGLA substantially, although it preferentially releases saturated and oligoenoic FAs. All together, these results suggested that compared to that of the other above $sPLA_2$s, the relative inefficacy of hGV to inhibit *Plasmodium* growth results from its inability to generate AA and/or other PUFAs in large amounts.

The anti-*Plasmodium* activity of human $sPLA_2$s is linked to their propensity to release PUFAs. Since arachidonic acid and other long-chain PUFAs are the NEFAs most active against *Plasmodium* (6, 45), we assessed whether the relative inefficacy of hGV $sPLA_2$ at inhibiting the parasite might result from its low ability to release PUFAs compared to that of hGIIF $sPLA_2$. NEFAs were extracted from a pool of LDL and HDL previously hydrolyzed by hGV versus hGIIF and then tested for parasite inhibition. As shown in Fig. 6, NEFAs from hGIIF-hydrolyzed lipoproteins inhibited *Plasmodium* with a 3-fold-lower IC_{50} than that of NEFAs extracted from hGV-hydrolyzed lipoproteins (6.5 ± 0.3 μg/ml versus 17.8 ± 1.0 μg/ml), demonstrating that the molecular nature of the released NEFAs is essential to the toxicity of $sPLA_2$-lipolyzed lipoproteins and confirming the importance of PUFAs as active agents of this toxicity.

To finally demonstrate a clear role for PUFAs in $sPLA_2$ toxicity against *Plasmodium*, we incubated hGIIF, hGIII, hGV, and hGX $sPLA_2$s with a parasite culture in Albumax II in the presence of liposomes with phospholipids containing at the *sn*-2 position either OA alone or PUFAs such as AA and docosahexaenoic acid (DHA; 22:6). We observed that hGIIF, hGIII, and hGX were clearly inhibitory in the presence of PUFA-enriched liposomes but not OA-containing liposomes, whereas inhibition by hGV was more modest (Fig. 7).

DISCUSSION

There is more and more evidence suggesting that a subset of human $sPLA_2$s contribute to host defense against various types of pathogens, including bacteria (13, 16, 18, 20, 51) and viruses (52–54). We show here for the first time that up to four human $sPLA_2$s can inhibit the *in vitro* growth of *P. falciparum*, which raises the question of their possible *in vivo* contribution to host defense against infection by this parasite.

Mechanism of action of human $sPLA_2$s against *P. falciparum*. By using catalytically inactive hGX and specific small-molecule inhibitors, we first demonstrated that the inhibitory activities of hGX and hGV $sPLA_2$s rely on their intrinsic catalytic activities. This could not be achieved in the case of hGIIF and hGIII $sPLA_2$s because of the lack of potent active-site inhibitors for these enzymes (11, 26). We next analyzed whether $sPLA_2$s act by hydrolyzing PLs from RBC cellular membranes and/or plasma lipoproteins. We found that hGX, and to a lower extent hGV, hydrolyzed RBCs infected by *P. falciparum* but not healthy RBCs, whereas

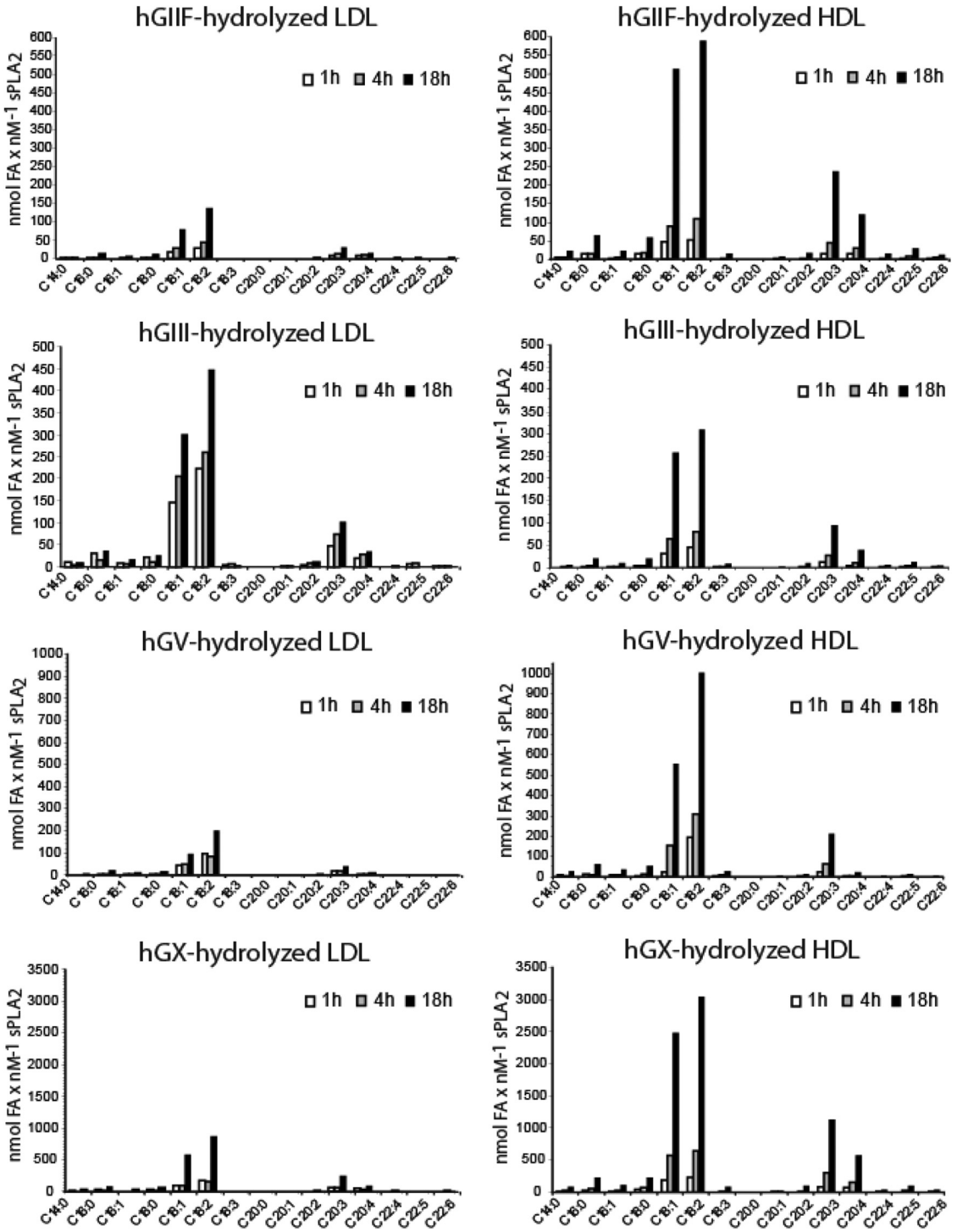

FIG 5 Lipidomic analyses of fatty acids released from LDL and HDL by human sPLA$_2$s. Purified human LDL (0.85 mg of PLs/ml) and HDL (1 mg of PLs/ml) were incubated at 37°C for 1 h, 4 h, or 18 h with recombinant human sPLA$_2$s. LDLs were incubated with hGIIF (75 nM), hGIII (45 nM), hGV (50 nM), and hGX (20 nM) sPLA$_2$s; HDLs were incubated with hGIIF (30 nM), hGIII (100 nM), hGV (13 nM), and hGX (6.5 nM) sPLA$_2$s. Lipids were extracted and processed for lipidomic analyses as described in Materials and Methods. Values were normalized according to the NEFA content in lipoproteins incubated alone.

hGIIF and hGIII sPLA$_2$s were poorly active on both types of RBCs. Among human sPLA$_2$s, hGX exhibits the highest catalytic activity on PC (11), the major PL species present in the external leaflet of mammalian cell membranes and lipoprotein surface. The capacity of hGX to hydrolyze external PC is governed by various factors, such as changes in membrane biophysics that can occur during cell infection, apoptosis, or necrosis, and also by the ratio of phospholipids to sphingolipids (50, 55). Such molecular determinants

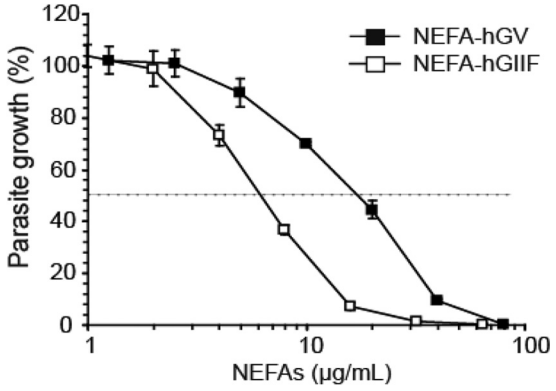

FIG 6 Role of NEFAs in *P. falciparum* inhibition by hGIIF- and hGV-hydrolyzed lipoproteins. A pool of LDL and HDL was lipolyzed by either 60 nM hGIIF or 50 nM hGV for 18 h at 37°C, and then NEFAs were extracted using FA-free BSA. The NEFA-loaded BSA was separated from the lipoprotein particle by ultracentrifugation, after which NEFAs were purified using the Dole's procedure and dried under N_2. After solubilization in RPMI medium–8% heat-inactivated plasma, NEFAs were assayed for inhibition of the FcB1 strain of *P. falciparum* in dose-response assays. NEFA-hGV, NEFAs extracted from hGV-hydrolyzed lipoproteins; NEFA-hGIIF, NEFAs extracted from hGIIF-hydrolyzed lipoproteins.

likely explain the selective action of hGX on infected RBCs. The membranolysis of infected RBCs by hGX $sPLA_2$ is also in line with the previously reported selective elimination of malaria-infected RBCs by a chemically modified form of pancreatic group IB $sPLA_2$ (5) that was attributed to impaired packing of PLs in the cellular membrane of infected RBCs and a facilitated membrane anchoring of the modified $sPLA_2$. The fact that membrane packing decreases during parasite maturation (39) likely explains the apparent selective action of hGX toward late trophozoites and schizonts.

Using conditions in which human plasma was replaced with Albumax II, we demonstrated that most of the anti-*Plasmodium* effect of hGX, hGV, hGIII, and hGIIF was dependent on the presence of plasma and, more precisely, lipoproteins in the parasite culture medium. This was reminiscent of the indirect mechanism of action of venom $sPLA_2$s (3, 4). However, the level of released NEFAs from plasma and lipoproteins by the different human $sPLA_2$s did not clearly parallel their anti-*Plasmodium* effects. In particular, hGIIF was 4-fold less active than hGV on plasma but 9-fold more potent at inhibiting *Plasmodium*. This indicated that the total activity on plasma of a given $sPLA_2$ (as measured by the release of NEFAs) is not the simple factor governing its toxicity.

When incubated at a rather low concentration (20 nM), hGIIF, hGIII, hGV, and hGX $sPLA_2$s induced toxicity of both LDL and HDL with the greatest efficiency. hGIII $sPLA_2$ induced the toxicity of LDL but had less effect than other $sPLA_2$s on HDL. These observations are in accordance with the relative activities of hGIIF, hGIII, hGV, and hGX at hydrolyzing lipoproteins, with hGIIF, hGV, and hGX showing preference for HDL over LDL and hGIII showing preference for LDL over HDL. This indicated that the different human $sPLA_2$s have distinct preferences for lipoprotein classes.

The rank order of hydrolytic potency of human $sPLA_2$s on lipoproteins was found to be hGX > hGV > hGIII and hGIIF > hGIB > hGIIA, hGIID, and hGIIE > hGXIIA (Table 2). These results are in accordance with previously published data (41–44). In comparison, the rank order of anti-*Plasmodium* toxicity was hGX > hGIIF > hGIII > hGV > hGIB, hGIIA, hGIID, hGIIE, hGXIIA, and hGXIIB in plasma. It could be noted that hGV is more potent than hGIIF at hydrolyzing total plasma and lipoproteins but less toxic at inhibiting *Plasmodium*. Since hGIIF $sPLA_2$ could not attack infected RBCs, a direct toxicity could not be involved in its higher efficiency against the parasite. A likely explanation for this apparent discrepancy was that the anti-*Plasmodium* toxicity of $sPLA_2$s depends not only on their capacity to hydrolyze lipoproteins efficiently but also on their specific enzymatic properties and capacity to release certain minor lipid products which are highly toxic to *Plasmodium*. We previously demonstrated that the main mediators of parasite death by bee venom $sPLA_2$-hydrolyzed VLDLs are PUFAs, especially AA, suggesting that the ability to produce PUFAs might be an important feature of the $sPLA_2$ anti-*Plasmodium* activity. It is known that hGX $sPLA_2$ preferentially hydrolyzes PC with polyenoic fatty acid species, including AA (41, 42, 46–48), and that hGIIF $sPLA_2$ exhibits preference for PC species with AA (44), whereas hGV $sPLA_2$ preferentially attacks oligoenoic PL species and mostly releases saturated and monounsaturated fatty acids (41, 42, 46–48). hGIII $sPLA_2$ did not seem to exhibit any PL preference (44). Our lipidomic analyses of NEFAs produced by hGIIF, hGIII, hGV, and hGX $sPLA_2$s from LDL and HDL were in line with those data. hGIIF, hGIII, and hGX

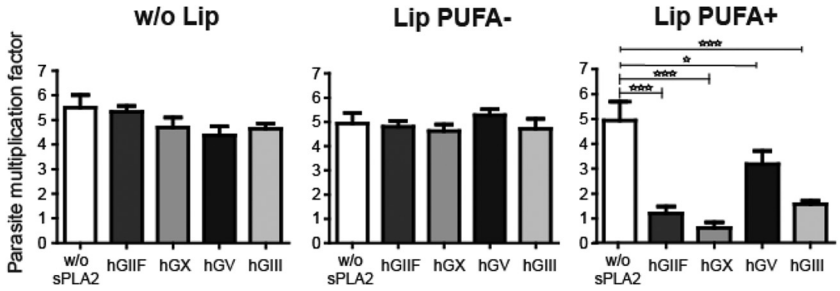

FIG 7 Involvement of PUFAs in the anti-*Plasmodium* activity of human $sPLA_2$s. A *P. falciparum* culture in 0.5% Albumax II was distributed in a 96-well microplate and supplemented with liposomes containing PLs with OA (Lip PUFA−) or AA and DHA (Lip PUFA+) at the *sn*-2 position or no liposomes (w/o Lip). hGIIF (40 nM), hGIII (45 nM), hGV (200 nM), and hGX (10 nM) $sPLA_2$s were added at time zero of incubation. Control for parasite growth was without $sPLA_2$ (w/o $sPLA_2$). Parasite multiplication was determined by optical examination of Diff-Quik-stained smears established before and after 48 h of incubation. Values are means ± SDs from 5 independent experiments. *P* values were determined from Friedman's test (with Dunn's multiple-comparison posttest) according to nonnormal sampling distribution. A *P* value of <0.05 (single star) was considered significant, and a *P* value of <0.001 (triple stars) was considered extremely significant.

$sPLA_2$s were all able to release substantial amounts of PUFAs, whereas hGV was much less efficient and, in particular, did not release AA. The most abundant fatty acids released by hGIIF, hGIII, and hGX $sPLA_2$s were 18:1, 18:2, 20:3, and 20:4 fatty acids, whereas hGV $sPLA_2$ released 18:1, 18:2, and 20:3 but not 20:4 fatty acids. However, hGV releases substantial amounts of DGLA (20:3), which suggested that its low selectivity for PUFAs might be restricted to PUFAs with more than three double bonds. Interestingly, hGIIF $sPLA_2$ was shown to exhibit the highest selectivity for PUFAs among $sPLA_2$s.

A prominent role of PUFAs in the anti-*Plasmodium* activity of lipolyzed lipoproteins was further substantiated through examination of the toxic capacities of the NEFAs extracted from hGIIF- and hGV-hydrolyzed LDL and HDL and by analyzing the toxic effects of liposomes enriched or not with PUFAs and treated with human $sPLA_2$s. First, the NEFA fraction from hGIIF-hydrolyzed lipoproteins was more toxic to *Plasmodium* than that from hGV-hydrolyzed lipoproteins, strongly suggesting that NEFA species specifically released by hGIIF but not hGV are responsible for toxicity. Given that hGIIF-derived NEFAs are enriched in PUFAs, especially arachidonic acid, a prominent role for PUFAs in hGIIF $sPLA_2$ toxicity and in other PUFA-releasing $sPLA_2$s could be proposed, reminiscent of what was observed with bee venom $sPLA_2$. Likewise, the relative inefficacy of hGV $sPLA_2$ at inhibiting *P. falciparum* growth despite substantial hydrolyzing activity on lipoproteins could be attributed to its inability to release AA and longer or more unsaturated PUFAs. Second, we observed that only liposomes enriched in PUFAs and treated with $sPLA_2$s that can release PUFAs, such as hGIIF, hGIII, and hGX, but not hGV $sPLA_2$s are toxic to *P. falciparum*. Our findings thus point to the fact that the human $sPLA_2$s that can release AA and other PUFAs are those with the strongest inhibitory effect on *Plasmodium*. Furthermore, our findings reveal a novel and remarkable activity of hGIIF $sPLA_2$ in this effect against *Plasmodium*.

In summary, the *in vitro* anti-*Plasmodium* toxicity of a given $sPLA_2$ results mainly from its specific abilities (i) to bind and efficiently hydrolyze lipoproteins and (ii) to specifically generate AA and likely other PUFAs that are, in turn, toxic to *P. falciparum*. Furthermore, the $sPLA_2$ selectivity toward lipoprotein classes (as exemplified by hGIII) and capacity to attack *Plasmodium*-infected RBCs (as exemplified by hGX) appear to be additional factors.

Expression and pathophysiological roles of human $sPLA_2$s in malaria. As far as we know, only two studies from the early 1990s have focused on the expression of human $sPLA_2$s in malaria. Both reported a significant increase in hGIIA $sPLA_2$ activity in the serum of infected patients, with a positive correlation to malaria severity (22, 23). However, only hGIB and hGIIA were known in the early 1990s, leaving open the possibility that additional $sPLA_2$s may be present in serum or infected tissues and hence may play a role in the pathophysiology of malaria. The serum concentrations of hGIIA can be as high as 100 nM in severe cases of malaria (22, 23). However, we show here that hGIIA is inactive against *P. falciparum* at concentrations as high as 1.5 μM under standard culture conditions, which suggests that its role in malaria, if any, might rather relate to inflammatory response of the host than direct inhibition of parasite development. However, it has been shown that hGIIA $sPLA_2$ exhibits enhanced potency toward HDL from acute-phase serum (56) and *in vitro*-oxidized lipoproteins (57), which raises the possibility that this $sPLA_2$ might be active against *Plasmodium* under pathophysiological conditions when lipoproteins get oxidized (58).

The expression and possible *in vivo* role of other human $sPLA_2$s in malaria are unknown. No thorough investigation to detect the different human $sPLA_2$s in the serum or tissues of malaria patients has been made. In *P. falciparum* malaria, it will be interesting to determine whether human $sPLA_2$s like hGX, hGIIF, hGIII, hGV, and hGIIA are present in the blood or at specific sites where infected RBCs are sequestered. Indeed, platelets and inflammatory cells (monocytes, lymphocytes, and neutrophils) have been observed along with the infected RBCs in brain capillaries of patients who died from cerebral malaria (59, 60), and hGV and hGX $sPLA_2$s have been detected in endothelial cells, macrophages, and neutrophils (61–63), leading to the possibility that these $sPLA_2$s are secreted locally and sequestered together with schizont-infected RBCs in brain capillaries. Acting in this microenvironment, the $sPLA_2$s would promote vascular endothelium activation involved in sequestration of parasitized RBCs (64), as well as destruction of mature parasites through hydrolysis of lipoproteins and/or elimination of infected RBCs. This view is supported by the fact that hGX $sPLA_2$-modified LDL can induce endothelial cell activation through V-CAM and ICAM-1 expression as well as adhesion of monocytes (65). A local action of hGIIF and/or hGIIA $sPLA_2$s should also be considered, since hGIIA $sPLA_2$ has been detected in platelets, mast cells, and macrophages (66, 67) and hGIIF has been detected in keratinocytes and endothelial cells (68).

In conclusion, we have shown that four human $sPLA_2$s (hGIIF, hGIII, hGV, and hGX) exert a potent *in vitro* anti-*Plasmodium* activity primarily by hydrolyzing lipoproteins from plasma, thereby releasing PUFAs that are toxic to the parasite. hGIIA $sPLA_2$, which has been found in the serum of malaria patients at elevated levels, was unable to kill the parasite under the same conditions, yet its role in a pathophysiological context of malaria infection remains to be investigated. Some of the above-listed human $sPLA_2$s might be endogenous factors acting against *Plasmodium*, yet the *in vivo* relevance of our observations remains to be established. Our studies further highlight a functional interrelationship between $sPLA_2$s and lipoproteins, which may act in concert in the pathophysiology of malaria, and possibly in other types of infections and diseases in which lipoproteins play a role (69–71). Human $sPLA_2$s with anti-*Plasmodium* activity might deserve future consideration as potential therapeutic tools or agents.

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